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Synergism between blue light and root exudate compounds and evidence for a second messenger in the hyphal branching response of *Gigaspora gigantea*

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Abstract: Light and chemical components of the host root exudate can induce hyphal growth and branching of arbuscular mycorrhizal fungi. Compounds that induce the same morphogenetic or biochemical response as light are referred to as photomimetic compounds (PCs). This is the first report of a synergistic response by *Gigaspora gigantea,* an arbuscular mycorrhizal fungus, to blue light and naturally occurring photomimetic compounds isolated from the exudate of host roots. The blue light treatment and exposure to photomimetic compounds were effective whether applied sequentially or simultaneously. The number of hyphal branches induced by blue light and photomimetic compounds together was greater than the sum of the branches generated by each separate treatment, and the synergism was greatest at the higher levels or orders of branches. The fact that blue light and PCs, individually, triggered the same hyphal branching response and when given together, they produced a synergistic response, indicated the activation of a second messenger in the induced-branching process. Delaying the application of PCs, after the initial light exposure, showed the second messenger was stable up to 3 h.

Key words: AM fungi, blue light, chemical signals, exudate, hyphal branching, photomimetic compounds, second messenger, synergism

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are obligately biotrophic organisms that form a symbiotic association with the roots of most land plants (Smith and Read 1997). The symbiosis is mutually beneficial because the fungus receives carbon compounds from the host (Pfeffer et al 1999) and the host receives greater vigor by increased uptake of soil nutrients that the fungus makes available to the host (Smith and Read 1997). The first interaction between fungus and host is the fungal reaction to chemical components or signals in the host root exudate. Host root exudates have a significant effect on the growth (Bécard and Piché 1989) and branching of AM fungi (Giovannetti et al 1993). The increase in hyphal branching induced by the exudate at or near the root surface (Giovannetti et al 1993, Nagahashi and Douds 2000) allows for greater physical contact between fungal hyphal tips and the epidermal cell walls of the host root, and this increases the chances of contact recognition and colonization of the host. Although the chemical nature of the hyphal branching stimulators is not known, there are a number of compounds in carrot root exudates that are active. Further separation of these compounds on silica gel G, TLC plates showed six different bands of activity (Nagahashi and Douds 2000). Whether there is only one chemical category with variable R groups or multiple chemical categories, has not been determined.

A recent report also showed that blue light can stimulate hyphal branching of AM fungi (Nagahashi and Douds 2003) and that this was ecologically relevant (Nagahashi et al 2000). Compounds or chemical agents that can induce the same biochemical or morphogenetic response as light have been referred to as photomimetic compounds (PCs) as reported earlier (Gressel and Rau 1983). For example, acetylcholine and eserine can induce conidiation of *Trichoderma viride* as does blue light (Gressel et al 1971). Also, *p*-chloromercuribenzoate in the dark can substitute for light in carotenoid synthesis in certain fungi (Rau et al 1967, Seviour and Read 1983).

However, little information is available on the interaction between light and naturally occurring photomimetic organic compounds. Specifically, do blue light (BL) and compounds from host root exudates interact to benefit the fungus? In this report, we have used a recently developed bioassay (Nagahashi and Douds 1999) to show that hyphal branching of *Gigaspora gigantea* can be synergistically stimulated with BL and semipurified PCs isolated from host root exudates. Further, these experiments demonstrate the

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existence of a second messenger in the hyphal branching response.

MATERIALS AND METHODS

Fungal spores.—Azygospores of *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe were produced in pot cultures in a greenhouse with *Paspalum notatum* Flugge as host. Spores from 8 mo old cultures were collected, isolated, sterilized, and aseptic spores were germinated on solid gellan (Phytagel, Sigma) medium (0.4%, W/V) in square (100 mm × 100 mm × 15 mm) Petri plates (Bécard and Fortin 1988). Each germinated spore was removed in a small plug and transferred to a fresh plate. One germinated spore was transferred to each new plate except where described below. Each plate containing a germinated spore was placed on edge for 3 d in the dark in an incubator at 32 C in 2% CO_2 before any light exposure or application of PCs. On the second day, the Petri plates were laid flat with the bottom side up for approximately 2 h. This allowed the primary germ tubes of *G. gigantea* (which exhibit negative geotropic growth) to grow toward the bottom of the plates. The plates then were placed on edge so that the primary germ tubes would turn at a right angle and grow vertically along the bottom of the Petri plate.

Root culture and concentration of root exudates (PCs).—Ri T-DNA transformed carrot roots (*Daucus carota* L.) were cultured in Petri plates containing M medium solidified with 0.2% gellan gum as described earlier (Bécard and Piché 1992). Roots were removed gently from the plates and transferred aseptically to sterilized M medium without gellan and grown in liquid culture 28 d as described (Nagahashi and Douds 2000).

The culture solution containing the root exudates was decanted and filtered through Whatman No. 1 filter paper to trap small root pieces, root caps and border cells. The fresh weight of roots was approximately 20 g per flask (250 mL of exudate/flask). The exudate was concentrated via SEPAK C18 cartridges (0.5 g) and eluted first with 3 mL of 35% acetonitrile followed by 4 mL of 70% acetonitrile. The 70% acetonitrile fractions were combined and dried under a stream of N_2. The dried sample was dissolved in a small volume of 70% methanol, diluted to 250 mL with de-ionized distilled water and concentrated in a fresh SEPAK cartridge. The final cartridge was eluted with 1 mL of 30, 40, 50, 60, 70 and 100% acetonitrile. The 60 and 70% fractions contained the most active fractions and were combined, dried under N_2, and dissolved in a ratio of 1 mL of 70% ethanol or methanol per every 250 mL of original exudate. The semipurified, concentrated exudate components (PCs) were diluted with 70% ethanol or methanol to achieve these dilutions: 1:100, 1:1000, 1:2000 and 1:5000.

Sequential application of high intensity BL and PCs using the microinjection assay.—To sequentially apply BL and PCs, the intensity of BL and concentration of PCs, which individually induced minimal branches, was determined first. To test both together, the BL treatment was given first (without the presence of PCs) followed by the immediate application of dilute PCs. Exposure with blue light was usually 5 or 10 min at 150 μmol s^{-1} m^{-2} in conjunction with four different PC dilutions. In one experiment, the time of application of PCs after BL exposure was delayed up to 6 h.

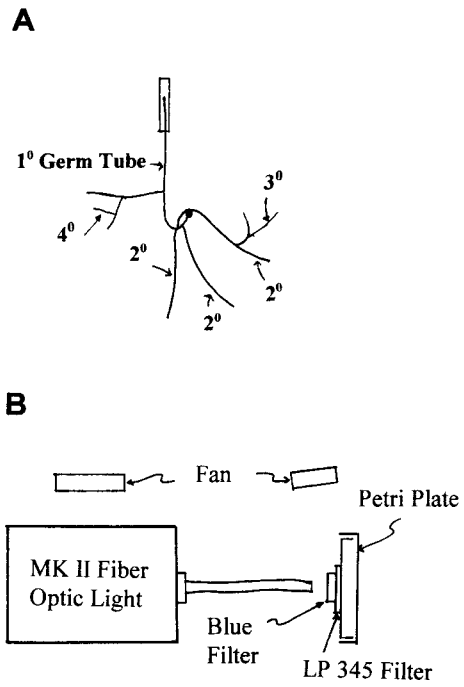

FIG. 1. A. Schematic drawing of the orders of hyphal branching observed during the experiments. The primary germ tube (1°), secondary (2°), tertiary (3°) and quaternary (4°) hyphae are labeled. The rectangular slit shows the part of the germ tube exposed to high intensity blue light for a short time. B. Schematic drawing showing the light source, filter placement, orientation of the fiber optic probe with a Petri plate and placement of cooling fans.

To test PCs from the exudate, a microinjection assay was used (Nagahashi and Douds 1999). Alcohol-sterilized Pasteur pipettes were fitted with cotton plugs and rubber bulbs and used to make two small sterile holes in the gellan 2 mm from the tip of a 3 d old germ tube. The pipette tip was inserted into the gellan, and the contents were removed by suction. The holes then were filled with 5 μL of PCs by microinjection with a Gilson-Rainin pipetman (P20) fitted with a fine application tip (Nagahashi and Douds 1999). The microinjected plates were placed on edge and incubated at 32 C in a 2% CO_2 for 16 h. Controls were injected with 70% ethanol or methanol (Nagahashi and Douds 2000). *Gigaspora* species usually have a single primary germ tube growing out of a germinated spore in a negatively geotropic direction. For the purpose of recording the level of branching off the primary germ tube, all branches arising from the primary germ tube are called secondary, those arising from secondary are termed tertiary, those arising from tertiary are quaternary (FIG. 1A).

For photo-induction of hyphal branching, light from a fiber optic probe (tungsten-halogen lamp) was passed through two filters and an aluminum foil slit. A 3-mm thick LP (long pass) 345 nm filter was used to block harmful UV

TABLE I. The effects of 70% ethanol on the blue light stimulation of hyphal branching of germ tubes of *Gigaspora gigantea*. Blue light exposure was for 5 min at 150 μmol s^{-1} m^{-2}. The light exposure was performed before the alcohol treatment (sequentially). Either 70% ethanol or methanol could be used. After the light exposure and alcohol treatment, all plates were placed in a dark 2% CO_2 incubator for 16 h at 32°C before branches were counted[x]

	Order of branches			
Treatment	Secondary	Tertiary	Quaternary	Total branches
Blue Light	1.30 ± 0.21a	0.60 ± 0.22a	0	1.90 ± 0.38a
BL + ethanol	1.40 ± 0.16a	0.70 ± 0.21a	0	2.10 ± 0.23a
Dark	1.30 ± 0.30a	0a	0	1.30 ± 0.30a
Dark + ethanol	1.20 ± 0.20a	0.10 ± 0.10a	0	1.30 ± 0.26a

[x] Means of 10 observations ± SEM. Means in the same column followed by the same letter are not significantly different (Tukey's Method of Multiple Comparisons, $\alpha = 0.05$).

rays below 345 nm and transmit all longer wavelengths and a 4 mm thick blue light filter was used to reduce light transmitted above 480 nm. Maximum transmission of light through the filters occurred at 390 nm. The filters were placed over a piece of aluminum foil that was taped to the Petri dish. The foil had a rectangular slit 8 mm long and 1.5 mm wide and was centered over the germ tube tip (FIG. 1A). The plate was set on edge and illuminated with a fiber optic probe set perpendicular to the filters. The intensity of blue light passing through the dual filters and one layer of the Petri plate plastic was 150 μmol s^{-1} m^{-2}, as measured with an IL 1700 research radiometer, calibrated at 390 nm, with an SED 033 detector (International Light, Newburyport, Massachusetts 01950-4092). Although the thick filters acted as a heat shield from the optic light probe, a fan circulated air around the Petri plate to minimize temperature fluctuations (FIG. 1B). For these experiments, two germinated spores were transferred to each plate. After 3 d in the dark, one germ tube of a germinated spore was exposed as above and the second was covered with aluminum foil and served as the unexposed, dark control.

Simultaneous application of PCs and BL (high intensity and low intensity).—For one type of simultaneous treatment, PCs were injected into holes in solidified medium and the plates were exposed immediately to high intensity blue light. In all of the experiments mentioned so far, only germ tubes with no branches in the apical 8 mm were selected and only new branches between the exposed area and the growing tip of the germ tube were counted. After any given treatment, the plates were incubated in the CO_2 incubator for 16 h, and the branches either were counted directly or the branching pattern was traced on the plate (Nagahashi and Douds 1999).

To rigorously examine effects of the simultaneous application of PCs and BL, an alternative method also was used. The PCs were mixed into the M medium before gellation (1 mL of concentrated exudate into 1 L of medium), and this mixture was poured into Petri plates. The control plates (M medium only) and the PC containing plates were exposed to BL at high (short term) and low intensity (long term, 10 h) before germinated spores were transferred to the plates. This approach let us assess the effect of BL on the M medium itself or its effect on the PCs directly. For these experiments, we counted all of the branches on the primary germ tube between the apical tip and the plug. All transferred spores were allowed to grow 3 d (72 h) before counting the branches. The synergistic stimulation was determined by letting the spores grow 46 h in the presence of PCs before the 10 h light exposure (0.8 μmol s^{-1} m^{-2}), and the branches were counted 16 h after the end of the exposure time (72 h total).

RESULTS

By using the microinjection bioassay, we could not easily distinguish possible photochemical changes in the growth medium components or photochemical changes in PCs, which could affect branching if we applied chemical compounds and exposed them to light simultaneously. The primary germ tubes were first exposed to light and then immediately treated with chemical signals (sequentially) and thus, the unexposed dark control, light only control and PCs only control were easy to monitor. Because the PCs were dissolved in 70% ethanol, a series of controls with and without 70% ethanol were performed (TABLE I). Ethanol or methanol (70%, Nagahashi and Douds 2000) did not stimulate or inhibit hyphal branching in the dark or in the light (TABLE I).

For these experiments, the germ tubes were exposed to blue light for 5 min (150 μmol s^{-1} m^{-2}) followed by treatment with dilute chemical signal (1:5000, v/v). The results (TABLE II) showed that, when the number of hyphal branches induced with blue light alone and dilute chemical signal (1:5000) alone were added, the sum (3.91) was considerably less than the number of branches generated with light and exudate together (10.67). The synergistic response was greatest at the higher orders (tertiary and quaternary) of branches. With the 5 min exposure to light but a more concentrated exudate (1:1000 dilution), the synergistic stimulation of hyphal branching was again greatest when comparing tertiary and

TABLE II. Synergistic effects of the sequential treatment of blue light and photomimetic compounds (PC) on hyphal branching of primary germ tubes of *Gigaspora gigantea*. The blue light exposure was for 5 min (BL = 150 μmol s^{-1} m^{-2}) and the semipurified exudate (PC) was diluted to 1:5000 or 1:1000 with 70% ethanol. The light exposure was performed before addition of PC and then all plates were placed in a dark CO_2 incubator for 16 h at 32°C before the branches were counted[x]

	Order of branches			
Treatment	Secondary	Tertiary	Quaternary	Total branches
BL	1.08 ± 0.23b	0b	0b	1.08 ± 0.23b
PC (1:5000)	1.83 ± 0.21b	1.00 ± 0.28b	0b	2.83 ± 0.31b
BL + PC	4.17 ± 0.42a	4.83 ± 0.56a	1.67 ± 0.31a	10.67 ± 0.94a
BL	1.08 ± 0.23c	0c	0b	1.08 ± 0.23c
PC (1:1000)	3.00 ± 0.23b	3.28 ± 0.32b	0.56 ± 0.17b	6.83 ± 0.47b
BL + PC	5.17 ± 0.29a	6.61 ± 0.49a	3.06 ± 0.39a	14.83 ± 0.80a

[x] Means of 12 observations ± SEM for top half of the table. Means of 18 observations ± SEM for the bottom half of the table. Means in the same column, within a group of three, followed by the same letter are not significantly different (Tukey's Method of Multiple Comparisons, $\alpha = 0.05$).

quaternary branches (TABLE II). At this dilution, the hyphal branching response for the PCs alone, as expected, was greater than with the 1:5000 dilution. The synergism was noticeable, although at this concentration of PCs (1:1000) the synergistic response (in terms of total branches) was not as great (less than twofold) as compared to the more dilute (1:2000, FIG. 2) or very dilute PCs (1:5000).

Increasing the light exposure to 10 min increased the number of branches (data not shown) when compared to the 5 min exposure. When this exposure time was coupled to concentrated PCs (1:100 dilution), the synergistic effect was still apparent although diminished compared to the previous experiments. Under these conditions, the synergistic stimulation was observed even at the 5th order of branches (data not shown). However, the more the PCs were concentrated, the greater the order of branching, the greater the total number of branches and the branches became smaller and difficult to count. Synergism with BL was difficult to quantify under these conditions.

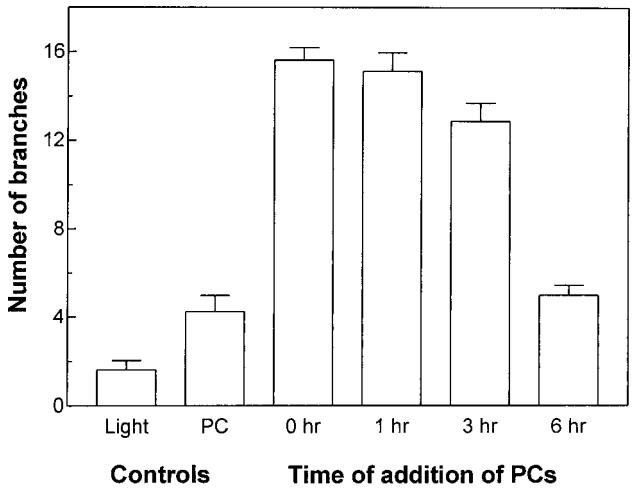

FIG. 2. Delay of the application of photomimetic compounds to the primary germ tubes of *Gigaspora gigantea* after the initial treatment with blue light. The initial light exposure was 150 μmol s^{-1} m^{-2} of blue light for 5 min and the exudate (PCs) was diluted 1:2000. Noted times are when the PCs were applied after the exposure to light. Each bar represents the means of 10 observations ± SEM.

For all of the experiments reported so far, the PCs were applied immediately after exposure to blue light. To determine if the time of application after exposure was important, PCs were applied at various times up to several hours after the initial exposure. If the PCs were applied within 1–3 h of the exposure, the synergistic response was not effected (FIG. 2); however, synergistic responses were not observed if PCs were applied 6 h after exposure.

Another experimental approach was used because adequate controls could not be included if we exposed the germ tubes to PCs and light simultaneously using the microinjection technique. For this experiment, 1 mL of the concentrated PCs was added to 1 L of M medium just before the medium solidified. This provided an even distribution of PCs in the plate, unlike the previous experiments in which the PCs were injected in small holes thereby creating an immediate concentration gradient. Controls, unamended plates and PC plates then were exposed to blue light for 10 h at 0.8 μmol s^{-1} m^{-2}. A germinated spore then was transferred to each plate. The total number of branches off the primary germ tubes after 72 h growth in untreated M medium was the same as for germ tubes growing in M medium pre-exposed to blue light (3.4 versus 3.1; TABLE III). This experiment also confirmed that blue light had no effect on PCs in regard to hyphal branching (16.3 versus 18.3; TABLE III) (i.e., the blue light acted upon the germinated spore to stimulate branching, not upon the

TABLE III. Determination of photo-induced changes in the media or exudate components (PC) that may affect branching of germinated spores of *G. gigantea* when blue light and PC are given simultaneously[x]

Treatment	Total branches
Spore added after treatment combination[y]	
Media in dark	3.4 ± 0.4c
Media + PC in dark	16.3 ± 1.2b
Media + 10 h blue light	3.1 ± 0.4c
Media + PC + 10 h blue light	18.3 ± 1.2b
Spore added before treatment combinations[z]	
Media + 10 h blue light	4.3 ± 0.3c
Media + 46 h PC + 10 h blue light	84.4 ± 6.2a

[x] Means of 8 observations ± SEM. Numbers followed by the same letter are not significantly different (Tukey's method of multiple comparisons, $\alpha = 0.05$).

[y] Control plates ± PC were kept continuously dark. Experimental plates ± PC were exposed to blue light for 10 h. Spores then were transferred to plates and grown in the dark at 32°C in 2% CO_2 atmosphere for 72 h.

[z] Spores grown for 46 h in the dark ± PC, then 10 h of blue light (0.8 μmol s^{-1} m^{-2}), and branches counted after a further 16 h growth (72 h total) in the dark at 32°C with 2% CO_2.

medium or PCs). To test for synergism, spores were grown 46 h in the CO_2 incubator in the presence of PCs before exposure to blue light for 10 h. After exposure, the plates were transferred back to the CO_2 incubator for an additional 16 h and the branches were counted (total of 72 h). The results showed a clear synergistic response (4.2-fold increase) between the PCs and blue light compared to the sum of the individual treatments (TABLE III). This experiment gave the greatest synergistic response because the hyphal branching response was primed by the presence of dilute PCs for 46 h before the light exposure.

This experimental approach then was performed with high intensity blue light (150 μmol s^{-1} m^{-2}) with a short exposure (5–30 min) in the presence and absence of PCs. A short exposure to high intensity BL had no effect on the medium components or PCs with regard to hyphal branching (data not shown). This result let us inject PCs and simultaneously give a short exposure to high intensity BL using the microinjection assay (TABLE IV). The results were almost identical to the sequential application approach shown earlier (TABLE II).

DISCUSSION

Earlier reports showed that light (Nagahashi et al 2000, Nagahashi and Douds 2003) and root exudates (Buee et al 2000, Nagahashi and Douds 2000) stimulate hyphal branching of germinated spores of *Gigaspora* species. This work showed that individual treatments of low levels of BL and diluted PCs were ineffective in stimulating hyphal branches but, when given together, a synergistic response was observed (TABLES II and IV). The BL exposure and PCs treatment can be given sequentially or simultaneously as shown by comparing TABLES II and IV, therefore, all future work can be done with the rapid microinjection bioassay.

A longer exposure to low intensity BL would be more meaningful physiologically. The results shown in TABLE III are physiologically relevant because they were performed with low levels of light (0.8 μmol s^{-1} m^{-2} for 10 h) and diluted PCs. The penetration of light through 3–6 mm of soil at fluence rates of 0.1 μmol s^{-1} m^{-2} or greater can stimulate biological responses (Bliss and Smith 1985, Tester and Morris 1987); thus the low light intensity used here was appropriate. The long exposure with low light intensity was used to mimic day length and, in the presence of diluted PCs, the results indicated that hyphal branching of AM fungi would be greater than that predicted to be caused by the individual effects of limited penetration of light and low levels of exudates from seedling roots growing near the soil surface. Under these conditions, a fourfold increase in

TABLE IV. Synergistic effects of the simultaneous treatment of blue light (BL = 150 μmol s^{-1} m^{-2} for 5 min) and dilute PC (1:5000) on the hyphal branching of *Gigaspora gigantea*. In this experiment, the exudate (PC) was applied and the plates were immediately exposed to blue light (simultaneous exposure to blue light and PC). After treatment, all plates were placed in a dark CO_2 incubator for 16 h at 32°C before the branches were counted[x]

Treatment	Order of branches			Total branches
	Secondary	Tertiary	Quaternary	
BL	1.30 ± 0.30b	0.30 ± 0.15b	0b	1.60 ± 0.30b
PC	2.00 ± 0.30b	1.20 ± 0.20b	0.20 ± 0.13b	3.40 ± 0.30b
BL + PC	5.00 ± 0.50a	5.30 ± 0.50a	2.10 ± 0.20a	12.40 ± 0.80a

[x] Means of 10 observations ± SEM. Means in the same column followed by the same letter are not significantly different (Tukey's Method of Multiple Comparisons, $\alpha = 0.05$).

hyphal branching was observed. This experiment was performed with only a 1 d cycle of light exposure and most certainly would have shown even greater differences if repeated 10 h light exposures were given on consecutive days. Because hyphal branching is the first observable recognition response between AM fungus and host root, any proliferation of hyphal branches increases the frequency of fungal contact with the root surface (Giovannetti et al 1993, Nagahashi and Douds 2000) and lets the fungus complete its life cycle via colonization of the root.

The fact that blue light and chemical signals (PCs) individually can induce hyphal branching suggested the activation or release of a second messenger. The synergistic stimulation under limiting conditions of low intensity blue light and low concentration of PCs confirmed the involvement of a second messenger. By delaying the time at which the PCs were applied after exposure to blue light, we have provided a way to investigate the stability of the second messenger. When the PCs were applied within 1–3 h after exposure, the synergistic response was apparent (FIG. 2). However, synergism was not observed after a 6 h delay, and this might indicate the turnover time for the second messenger. It remains to be determined whether light and chemical compounds have the same receptor or whether there are two separate receptors that initiate the same chain of events via the second messenger.

Photo-induction events and blue light/near UV light receptors have been studied in various fungi (Gressel and Rau 1983, Corrochano and Cerda-Olmedo 1991, Horwitz and Berrocal 1997, Lauter et al 1998, He et al 2002), but only limited work has been done with AM fungi (Nagahashi et al 2000, Nagahashi and Douds 2003). Although *G. gigantea* appears to be an ideal test organism for future molecular biology studies involving a blue light response, it is an obligate symbiont and has not yet been cultured axenically (without a host root). Consequently, large amounts of aseptic spores cannot be obtained readily. Perhaps in vitro culture techniques, such as those with other AM fungi (e.g., *Glomus intraradices* [St.-Arnaud et al 1996]), could be used to mass produce *Gigaspora* spores monoxenically (with host root) for biochemical and genetic studies.

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